Spinning skin into neurons

Basic helix–loop–helix proteins are transcriptional regulators with important roles in myogenesis and neurogenesis. One such protein, NeuroD, has an impressive ability to promote ectopic neuronal differentiation in *Xenopus*.

In the late 1980s, a remarkable convergence of discoveries led to the identification of transcription factors in the basic–helix–loop-helix (bHLH) family as critical cell-type determinants in metazoans. Almost simultaneously, bHLH proteins were uncovered by loss-of-function mutations affecting the genesis of neurons in the fruitfly [1], and by gain-of-function mutations affecting the genesis of muscle in the mouse [2]. In addition, bHLH proteins were identified in biochemical studies of mammalian B-cell development. Together, these discoveries allowed the formulation of a set of rules governing the interactions of bHLH proteins in multimeric complexes [3]. As the size of the bHLH family expanded, it became clear that its diversity subserves two distinct developmental functions: different bHLH proteins can act within a given lineage to control successive stages in the development of a particular cell type, and in different lineages to generate distinct but related cell types [4]. Moreover, at a given stage of development, bHLH proteins act in a genetic network that involves both stimulatory and inhibitory interactions between different family members [5].

The discovery that bHLH proteins are specifically expressed in the developing vertebrate nervous system [6] indicated that focusing on these proteins could lead to useful insights into vertebrate, as well as invertebrate, neurogenesis. Studies of bHLH protein functions in vertebrate neurogenesis have lagged behind those of vertebrate myogenesis, however, in part because of the difficulty of obtaining both loss-of-function and gain-of-function mutant phenotypes for a given gene within a single vertebrate system. For example, targetted inactivation of the gene *mammalian achaete-scute homolog 1* (*Mash-1*) in 'knockout' mice caused the elimination of peripheral autonomic and olfactory sensory neurons [7], but overexpression of *Mash-1* has thus far not yielded premature or ectopic neuronal differentiation in any mammalian system [8]. On the other hand, overexpression of *Xash-3*, a *Xenopus achaete-scute* homolog normally expressed at the time of neural induction [9], has been shown to cause an expansion of neural tissue at the expense of surrounding ectodermal tissue [10,11].

The recent description [12] of a striking gain-of-function mutant phenotype of a novel bHLH gene, *NeuroD*, in *Xenopus* has stirred interest in its product's function in vertebrate neurogenesis. Importantly, although its name might superficially suggest that NeuroD protein is a neural analog of the myogenic determination factor MyoD, a comparison of the NeuroD gain-of-function phenotype to that of Xash-3 suggests that NeuroD is more likely to play a role in neuronal differentiation than in determination.

In contrast to *Mash-1* and *Xash-3*, which were isolated by using the polymerase chain reaction (PCR) to clone vertebrate homologues of the *Drosophila achaete-scute* genes, *NeuroD* was isolated by a genetic screen in yeast designed to detect protein–protein interactions. This screen is especially applicable to bHLH proteins, because they can heterodimerize promiscuously with the ubiquitously-expressed bHLH proteins of the 'E protein' subfamily, such as E12/E47 or the product of the *Drosophila daughterless* gene (an interaction partner of *achaete-scute* products) [3]. In both mouse and *Xenopus*, *NeuroD* mRNA is transiently expressed by subsets of neurons just as they begin to differentiate [12]. However, *NeuroD* is expressed later than *Mash-1* and *Xash-3* in mouse and frog, respectively (Fig. 1); for example, in the murine hindbrain, *Mash-1* is expressed in the ventricular zone whereas *NeuroD* is not expressed until differentiating neuroblasts have migrated out of the ventricular zone. These descriptive data suggest that *NeuroD* functions at a later stage in neurogenesis than do the the two vertebrate *achaete-scute* homologues.

Three distinct phenotypes were observed in *Xenopus* embryos in which *NeuroD* was overexpressed [12]. First, a premature and more extensive differentiation of neurons was observed in the central nervous system. Second, a loss of non-neurogenic cephalic neural crest and an expansion of neurogenic cephalic neural crest were detected. Finally, an apparent conversion of non-neurogenic ectoderm to neurons was seen. Curiously, the timing of these effects was different: the neurogenic phenotype within the neural plate was observed at stage 12, whereas that in the epidermis was not detected until stage 19. This may reflect differences in the competence of these tissues to respond to ectopic *NeuroD* expression, as has been observed for ectopic *achaete-scute* expression in *Drosophila*. Overexpression of *NeuroD* caused the genesis of several different types of neuron, suggesting that it participates in a common neuronal differentiation program in several distinct neurogenic sublineages, rather than in the specification of a specific neuronal cell type.

The *NeuroD* gain-of-function phenotype was distinct from that of *Xash-3* in two important respects: ectopic *Xash-3* caused an expansion of the neural plate at the expense of surrounding ectoderm (Fig. 2a), whereas ectopic *NeuroD* increased neuronal differentiation within

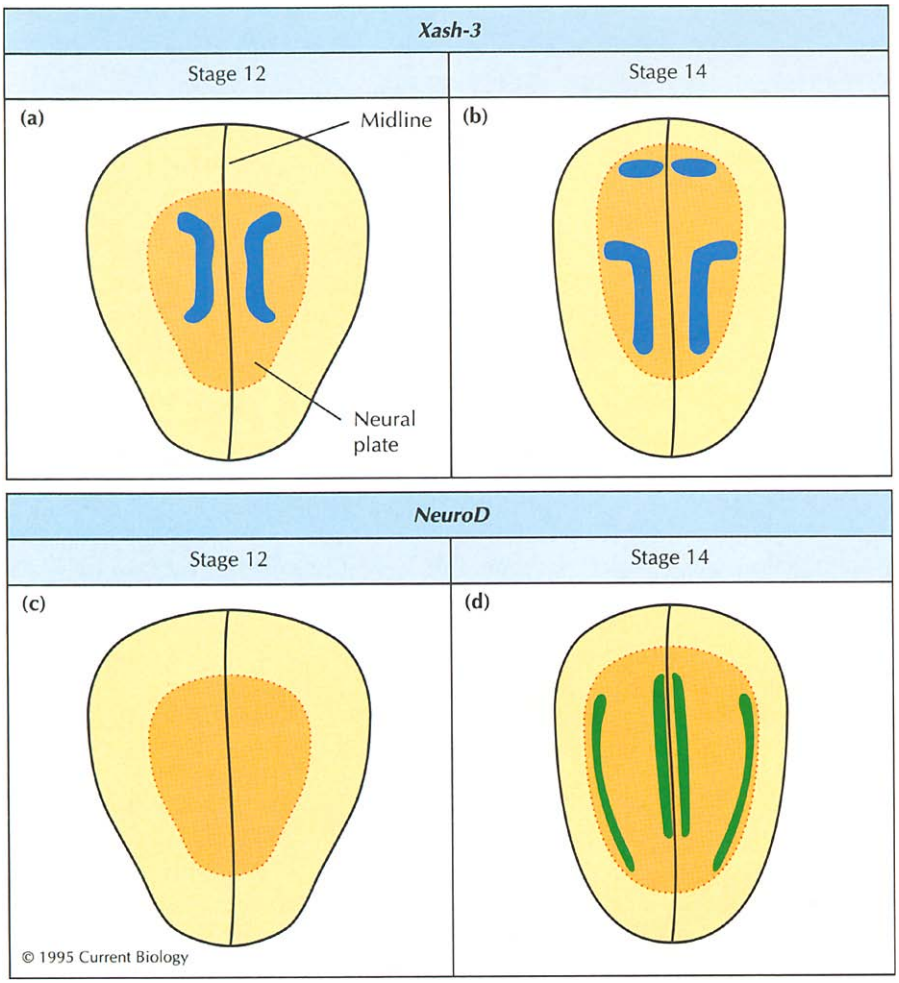

Fig. 1. Expression of endogenous *Xash-3* (blue) and *NeuroD* (green) mRNAs during early *Xenopus* development. Embryos at stage 12 express *Xash-3* **(a)** but not *NeuroD* **(c)** in the neural plate (orange), whereas at stage 14 both genes are expressed **(b,d)**, albeit in different and apparently non-overlapping patterns.

the neural plate but did not expand it (Fig. 2b). Conversely, ectopic *NeuroD* caused neuronal differentiation within the epidermis (Fig. 2d), whereas ectopic *Xash-3* did not (Fig. 2c). The neural plate data are consistent with the idea that NeuroD can accelerate neuronal differentiation in a cell population that is already determined. On the other hand, the ability of NeuroD to induce neurogenesis in cultured animal caps as well as in non-neurogenic ectoderm suggests that it can exert both a determination and a differentiation function when overexpressed. Nevertheless, the fact that NeuroD is expressed later than Xash-3 leads Lee *et al.* [12] to conclude that NeuroD normally functions in differentiation rather than determination.

Lee *et al.* [12] suggest that the reason NeuroD, but not Xash-3, can generate ectopic neurons in the skin when overexpressed may be because there are inhibitors of bHLH activity in that tissue to which Xash-3, but not NeuroD, is sensitive. The problem with this simple explanation is that the ability of NeuroD to induce ectopic neurons in the skin is restricted — only some of the skin cells expressing ectopic NeuroD become neurons. Lee *et al.* [12] suggest two possible explanations for this: ectopic NeuroD may activate a lateral inhibition process similar to that seen in *Drosophila* [13]; alternatively, neuronal conversion may be restricted by some kind of a prepattern in the skin, as indicated by the regularly-spaced distribution of ciliated epidermal cells. Whatever the reason, the action of NeuroD is clearly restricted in the skin, and it will be important to understand the basis of this restriction in order to define the activity of NeuroD.

Differential sensitivity of Xash-3 and NeuroD to inhibitors, if real, could support a potentially important distinction between 'determination' and 'differentiation' bHLH proteins, previously suggested by Weintraub [5] for myogenesis. Specifically, Weintraub proposed that determination factors, such as myf5 and MyoD, are more sensitive to inhibitory bHLH proteins, such as Id and Hairy, than are differentiation factors, such as myogenin and MRF4, because of structural differences between the proteins [5]. A similar distinction might hold for Xash-3 and NeuroD [12]. The advantage of such a system is that it permits control of determination by environmental factors that regulate the inhibitors [13]. Once the determination bHLH proteins reached a critical threshold, however, they might activate new bHLH proteins that would be insensitive to the inhibitors, thereby 'locking' the cell into a particular pathway of differentiation [5].

There are problems with applying this attractive model to Xash-3 and NeuroD, though. Firstly, these two bHLH proteins are not successively expressed in the same lineage,

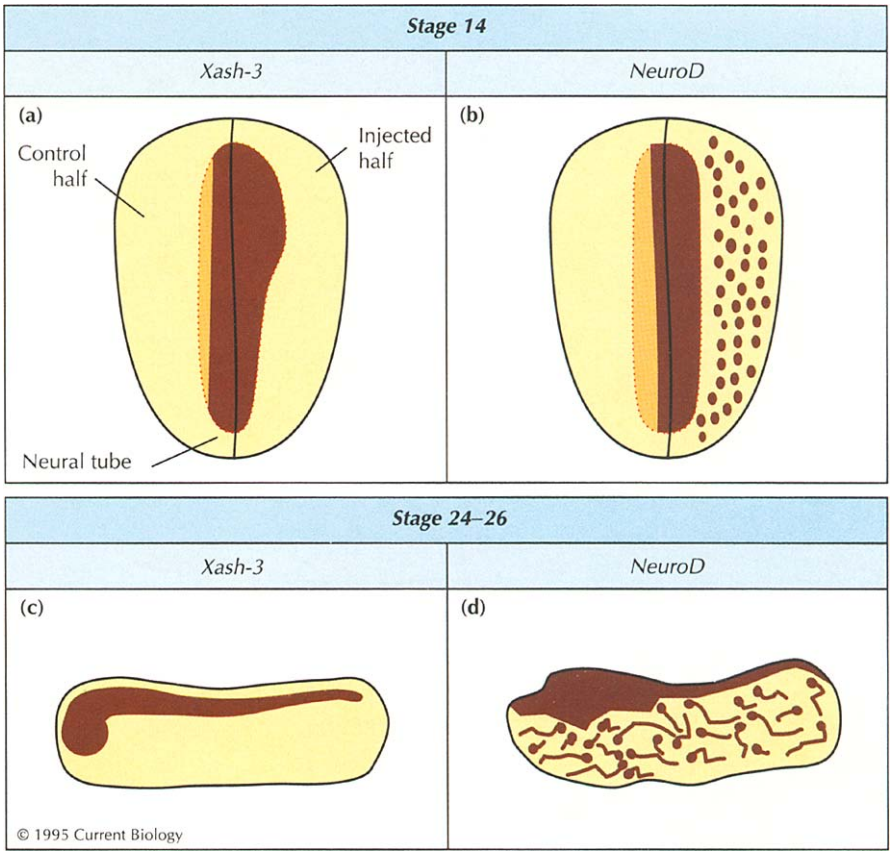

Fig. 2. Consequences of ectopic expression of *Xash-3* and *NeuroD* in *Xenopus* embryos, as assayed by expression of the neural cell marker N-CAM (brown). Embryos injected with either *Xash-3* **(a,c)** or *NeuroD* **(b,d)** mRNA are illustrated at either stage 14 (a,b) or stage 24–26 (c,d). In (a,b), the injected side of the embryo is to the right of the midline; the left side serves as a control. Note that *Xash-3* overexpression expands the neural tube (a), whereas *NeuroD* overexpression widens the domain of N-CAM expression within the neural tube, but does not expand it (b). Conversely, *NeuroD* overexpression causes ectopic N-CAM expression in subsets of ectodermal cells (d), whereas *Xash-3* overexpression does not (c).

but rather in different regions of the nervous system [9,12]. Their apparent differential sensitivities to inhibitors might therefore reflect more the lineages in which they are expressed than the stage of development that they control. Secondly, the inability of NeuroD to expand the neural plate could also be interpreted as reflecting a sensitivity to inhibitors, although clearly these inhibitors would have to be different than those operating in the skin.

In vertebrate myogenesis, there are at least four, and possibly five, different bHLH proteins that control sequential steps of muscle development [14]. There is no reason to think that neurogenesis should be any less complex. Indeed, at least one additional bHLH protein, NSCL1, appears to be expressed subsequent to NeuroD [15]. It may be premature, therefore, to try to identify unitary 'determination' and 'differentiation' steps within a lineage and assign them to specific bHLH proteins. Rather, the process of commitment at the molecular level may be a more gradual one, in which successively expressed bHLH proteins progressively restrict the developmental options of a precursor cell, perhaps by rendering it increasingly insensitive to environmental influences that could inhibit or alter its pathway of differentiation. In that case, the different bHLH factors that operate in a given lineage might be most usefully distinguished in terms of their thresholds of sensitivity to various inhibitors.

The functional analysis of bHLH proteins has been complicated by the fact that these proteins show both redundancy and promiscuity. On the one hand, multiple, redundant bHLH proteins may control a common developmental step; on the other hand, a single bHLH protein may adopt the function of a related protein if misexpressed. Ideally, therefore, conclusions about the normal function of a given bHLH protein should be based on both loss-of-function and gain-of-function manipulations within a given lineage, as has been possible in vertebrate myogenesis and fly neurogenesis. Unfortunately, loss-of-function genetic perturbations are presently difficult to accomplish in *Xenopus*. Loss-of-function perturbations for NeuroD are therefore most likely to be accomplished in the mouse, the vertebrate system that is best suited for gene disruption experiments.

Although the *Xenopus* data would lead one to predict that targetted inactivation of the *NeuroD* gene should prevent neuronal differentiation, an independent study [16] suggests that NeuroD may function in other tissues as well. Specifically, NeuroD was independently isolated in a yeast interaction screen for bHLH proteins that bind to the insulin promoter, where it was named BETA2 [16]. *BETA2/NeuroD* mRNA is expressed at high levels in pancreatic β cell lines, and BETA2/NeuroD can function as a strong transcriptional activator of the insulin promoter–enhancer in transient co-transfection experiments [16]. These results suggest that loss-of-function perturbations of BETA2/NeuroD may affect the pancreatic islet cells as well as the nervous system.

The study of bHLH proteins promises further opportunities to unravel the complex genetic circuitry that

controls cell-type determination in general, and neurogenesis in particular. The discovery of NeuroD has identified an important new player that may control an important late step in neuronal differentiation shared by many classes of neurons, in many vertebrate (and possibly invertebrate) organisms.

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David J. Anderson, Division of Biology 216-76, Howard Hughes Medical Institute, California Institute of Technology, Pasadena, California 91125, USA.